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IMMUNE-FLUORESCENCE: PRINCIPLES, PROCEDURES, APPLICATIONS

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DEFINITION

First developed and perfected by Coons, this technique involves joining globulin-antibody molecules with a fluorochrome; a fluorochrome is a substance which gives off a secondary light called fluorescence in response to a primary irradiation known as the "excitant."

This conjugation does not affect the immunological reactivity of the remaining antibodies, which are still capable of specific attachment to the corresponding antigens.

These antigens can also be found in specific proportions on slides under microscopic examination performed with lighting set at the excitant wavelength, by reason of the fluorescence of the antibodies affixed to them. At the same time, the antigens are identified, since the immunological specificity of the antibodies is known.

This the immune-fluorescence technique combines the ease and rapidity of microscopic examination with the specificity of a serological examination.

TECHNIQUES AND EQUIPMENT USED

1. Preparation of antibody colutions and conjugation with the fluorochrome.

-- Ordinary procedure does not call for purified antibodies; oftenest used are simple globulin solutions taken from the serum of a lab animal which has been hyper-immunized with the desired antigen. This globulin fraction is usually separated from the

albumens by fractionation with semi-saturated ammonium sulphate.

-- This globulin solution containing the antibodies and globulins is then linked with the fluorochrome.

The fluorochromes used are chemical derivatives of ordinary fluorochromes, chosen for their ability to combine with the amino radicals of the globulins. The secondary fluorescent light they give off is of a different color for each one, but it always has a longer wavelength than the primary light source.

The primary light source is kept in the ultra-violet, violet, and blue wavelengths for these fluorochromes, the commonest of which are the following:

§ Isocynate of fluorescein was the first fluorochrome used by Coons.

§ Far the commonest choice nowadays, since Riggs published his work, is isothiocyanate of fluorescein, which is more stable than the earlier compound. This substance apparently combines with the amino radicals of the globulins through its isothio bond (-N-C-S).

The fluorescence of these two fluorescein derivatives is greenish-yellow in color. The maximal intensity of its emission spectrum is somewhere around a wavelength of 550 millimicrons.

§ Lissamine-Rhodamine B gives off an orange-red fluorescence (2 emission peaks: 595 and 710 millimicrons).

§ 1-dimethylaminaphthalene-5-sulphonic acid, which has an emission spectrum in the yellow and green range (between 450 and 620 millimicron wavelength) is much less frequently used..

-- Conjugation is effected by agitation blending at low temperatures under very specific pH conditions (pH 9 - carbonate buffered). The amount of fluorochrome incorporated in the mixture is a function of the weight of globulins to be conjugated and the fluorochrome used. With the commonest fluorochrome, isothiocynate of fluorescein, the fluorochrome/globulin weight ratio generally chosen is 1 to 20. Under these conditions, the globulin molecules are tagged -- and this is an average, because the conjugation is not homogeneous -- with 6 molecules of isothiocyanate of fluorescine apiece.

-- But a great many fluorochrome molecules are not affixed to the globulins. This remainder is removed by various methods, the commonest of which are dialysis, charcoal absorption, and the general favorite, Sephadex gel filtration.

After various physical and immunological tests which we shall come back to later on, the solution of conjugated globulins and antibodies ("conjugate" for short) is bottled and stored in refrigerators.

2º ANTIGEN PREPARATIONS

These are slide-fixed preparations. They may be microbe cultures, pathological smear samples (stools, throat, LCR, etc.), or blood smears. They may also be cell cultures or histological sections. In the latter case, the sections are made under cryostat freezing, in order to avoid degeneration of the antigens.

Most frequently, the preparation is fixed. The choice of fixative depends on the antigen you are looking for, because the fixing agent must be one that will not seriously distort the antigen. One of the commonest, and one that works well in the majority of cases, is acetone, which is allowed to work for about ten minutes. However, ethanol and methanol are also quite often used. The life of such preparations under refrigeration varies widely from one slide to another.

3º OPTICAL EQUIPMENT

This requires an ordinary microscope with a wide-field lens, equipped with a diaphragm. For high magnification, use x-100 immersion lenses with a mineral oil which is not spontaneously fluorescent. Actually, though, powerful dry lenses (x63, x70) are often preferable. They are easier to handle, and their coarser resolution is of no importance when the purpose is to observe a fluorescent object. No lens used should be spontaneously fluorescent.

The examination may be made against a light field or a dark field, since either one has both advantages and disadvantages. The fields need not be quartz, thanks to the intensity of the light source.

The light source is a very powerful (usually 200-Watt) mercury vapor lamp, shielded by a metallic housing. An "excitant" filter placed in front of the lamp blocks the extraneous rays and admits only those which can be absorbed by the fluorochrome and which can trigger its fluorescence. In other words, under usual conditions, it will admit the rays from 320 to 480 millimicrons' wavelength (ultra-violet, violet, blue).

A second filter, known as the "stop" filter, is placed either at the level of the lens or in the microscope tube. It blocks the blue-violet and ultra-violet rays (the latter are

dangerous to the eyes), and allows only the fluorescence wavelengths to pass; these will be greenish-yellow or orange-red, depending on the fluorochrome chosen. Photographs, either in black-and-white or in color, may be taken with very sensitive film.

4° APPLICATION PROCEDURES

-- The direct method was the first one Coons used, and it is the simplest in execution.

Some time beforehand, a few drops of the conjugated antibody solution are placed on the prepared slide. Contact is maintained for 20 to 30 minutes. If the preparation contains the element you are looking for, the homologous conjugated antibodies will have fixed on the antigen sites of that element within that space of time. The slide is then carefully and gently washed by rinsing in buffered physiological water (pH 7) so as to get rid of the unattached antibodies, as well as to remove the globulins having no antibody activity. The preparation is then covered with a drop of buffered (pH 7) glycerine, and finally with a cover glass. It is then examined under the microscope under the optical conditions described above.

If the preparation does contain some of the bacteria you are looking for, they will stand out because of the fluorescent ring around them, produced by the fixation of numerous conjugated homologous antibodies around the antigen sites. They are easy to find, because their morphology is readily recognizable. Recognition is tantamount to identification, by reason of the immunological specificity of the reaction.

However, if the preparation contains only bacteria whose antigens do not correspond to the conjugated antibodies, they will remain invisible.

Bacteria are not the only forms that can be identified in preparations consisting of cultures or of pathological smears: it is also possible to identify parasites, fungi, and viruses. For viruses, the preparation generally consists of a layer of a cell culture or of a histological specimen of the infected tissue. The viral antigens appear in the form of clumps of fluorescent dots in the cytoplasm or in the cell nuclei. It is also possible to find antigens in the molecular state in histological sections or in cellular smears.

2 -- The Indirect Method is based on the principle of the Coombs test, also known as the "sandwich" method.

This procedure is most often used to look for antibodies in a serum specimen, by means of a known antigenic preparation.

It is done in two steps:

-- In the first step, a few drops of the serum under study are placed on the slide preparation of the antigen, and allowed to work for 20 to 30 minutes. The preparation is then washed in physiological water, just as was done in the direct method, in order to get rid of the non-fixed antibodies and the other serum elements.

-- In the second step, a few drops of a solution of conjugated antibodies -- antigammaglobulins from the species used for the serum in the initial layer under study -- are placed on the preparation. These tagged antibodies will take hold during contact -- again a period of 20 or 30 minutes -- of the first layer of gamma globulins already fixed on the antigens. Actually, the antigens behave like antibodies towards the antigens in the preparation, and like antigens towards the fluorescent antibodies in the second layer. A second rinsing will wash away any unfixed conjugated antibodies. The preparation is then mounted and examined as above.

One practical example will suffice to illustrate this indirect procedure in IF as a tool for serological study: let us take the case of syphilis. The antigen preparations are slide smears of Treponema pallidum. During the first step, the serum under study is placed on a slide -- or rather, several slides are used with several different dilutions of this human serum, thus making the procedure quantitative as well. In the second step, we add the conjugated human antigammaglobulins, usually prepared from the serum of rabbits hyperimmunized with the Cohn fraction of human serum. If the human serum tested contains antibodies, the treponemes will show up as fluorescent, with all their typical morphology, and the quantitative threshold can be determined; if there are no antibodies, the treponemes will remain invisible.

However, the indirect IF procedure is not confined solely to the detection of serum antibodies. It can also be used to detect antigens in the preparation. In this case, the first layer will consist of a hyperimmunized antiserum of known specificity, and, since this will generally be a rabbit serum, it is a conjugated (with goat or duck) rabbit antigammaglobulin which is added in the second step. The advantage of this technique is that one need prepare and use only one conjugate in seeking several microorganisms.

Another original application of indirect immune fluorescence, with some modification of the principle, consists in pinpointing tissue antibodies on the level of the immunity-producing cells, for example, by using a solution of corresponding antigens in the first step, and a solution of antibodies identical with the ones you are looking for, conjugated, in the next.

3 -- The fluorescence inhibition method suggested by Goldman consists in bringing the serum to be tested into contact with known antigens. If the serum contains the antibodies you are looking for, they will fix specifically on the antigens, and prevent the fixation of a conjugate solution of identical antibodies which are added in the second step. The reaction is positive when there is no fluorescence observable in the antigen preparation.

4 -- The anticomplementary method (Goldwasser and Shepard) uses a solution of anticomplementary fluorescent guinea-pig antibodies designed to make visible any immune compounds upon which the substance fixes. This is thus a variation of the serological reaction of complement fixation.

All these elegant procedures make it possible to use immuno-fluorescence in a great many theoretical and practical problems of microbiology and immunology. However, it would be erroneous to assume that immuno-fluorescence is always simple and reliable in application. It has its drawbacks and its limitations, which must certainly be pointed out.

DRAWBACKS AND LIMITATIONS OF IMMUNE-FLUORESCENCE: CHECKS TO BE PERFORMED

The IF technique must be considered as a serological method, since it involves an immune reaction.

a) The sensitivity of the technique is good. In the direct procedure, it can reveal very small quantities of antigens, such as a few bacteria, even if they are killed and cannot be cultured. In the indirect serological procedure, it is considered more sensitive than precipitation, complement fixation, neutralization, or agglutination, though less sensitive than conditioned hemagglutination or the Ovary test.

This very good sensitivity, however, is a function of proper immunization of the animals supplying the serum. The use of immunization boosters is often helpful in obtaining this. Every new conjugate used should be titred for sensitivity.

b) The specificity is highly variable from case to case. In general, it is not very good, for several reasons:

§ While the technique has the advantages of immunological specificity, it also has its limitations, even here. The antigen communities so frequently encountered in microbiology are actually responsible for mixed reactions. These are even more frequent than with other procedures, apparently for two reasons:

-- The great sensitivity of the method increases the chances of mixed reactions; we might cite, for example, the one observed for A, C, and G-group streptococci, which are far less evident with less sensitive techniques.

-- On the other hand -- and this holds true primarily for bacteria -- fixation makes the underlying antigens "available," as well as the often more specific superficial antigens, which are the only ones involved in other reactions such as agglutination and immobilization. For example, the protein of the treponeme group is involved in indirect IF of syphilis, whereas only the specific antigens of Treponema pallidum are involved in the Nelson test.

And this is why, very often, these crossed reactions in IF cannot be predicted on the basis of classic serological procedures. This means that they must be carefully tracked down and prevented by means of numerous tests and checks, but we cannot go into the details of these procedures now.

In order to get around this difficulty, we use the maximal dilution of the serum or conjugate which will give us optimal fluorescence. We make a particular effort to eliminate the antibodies responsible for mixed reactions by means of attrition techniques. For example, attrition of antistreptococcus A conjugate with streptococcus C will eliminate mixed fluorescence. Attrition of syphilitic serums with Reiter Treponemes, which are not pathogenic, but which do contain the group protein, seems to eliminate some of the non-specific fluorescents observed in low dilutions.

However, these latter procedures are not always adequate, and the degree of mixed or cross-reactions sometimes makes IF inapplicable; it fails, for example, in enteral infections of Salmonellae and Shigellae, because of their antigen community with the other enterobacteria.

§ Along with these immunological failures of specificity, we often observe non-specific fluorescence of a different kind, which must also be controlled. Apparently this is due:

-- to the presence of numerous "natural" antibodies in the serums (as opposed to bacterial or tissue elements and the like), which is again heightened by the great sensitivity of IF.

-- to the visible tendency of the serum proteins, particularly the globulins which have been slightly altered by conjugation, to adsorb on the cell or tissue elements of the fixed preparation.

These suggestions have been made to get around these two problems:

§ attrition of the conjugates with powdered organ tissues (liver, placenta...)

§ optimal tagging of the globulins used. The number of molecules of fluorochrome conjugated with each molecule of globulins is important here. If it is too low, it lowers the sensitivity of the conjugate; if it is too high, it lowers its specificity. The optimal figure for bacteriological purposes would seem to be about 6/1. On the other hand, in histological applications, a lower ratio (2/1) is preferable. This conjugation proportion must be figured in the conjugate on the bases of the amount of globulins on the one hand and of fluorochrome on the other (measured under the spectroscope). Under the usual conditions of conjugation, this proportionality is an average: molecular tagging is not homogeneous. It is possible to select a homogeneous batch of tagged globulin molecules in the desired proportion by chromatography on a DEAE cellulose strip. Finally, the quality of the conjugates can be still further improved if you use a purified solution of conjugated antibodies, one which contains no non-antibody globulins for the desired specificity. Certain very delicate techniques now make it possible to do this.

c) The IF technique is not perfectly patient of reproduction. The results it yields are actually the combined function of a number of factors, including the antigen preparations, their mode of fixation, and, above all, the characteristics of the conjugates. Thus far, the conjugates are not standardized. Commercially obtainable conjugates are very uneven in quality. The intensity scale for fluorescence, generally indicated as + to +++, is subjective and altogether arbitrary.

d) The rapidity of the IF technique is common knowledge. This is one of its greatest advantages. However, the indirect procedure, which takes twice as long as the direct one and requires the preparation of several dilutions, is fairly cumbersome. Furthermore, only a limited number of slides can be examined at a sitting, because of eye fatigue.

The advantages and disadvantages of IF, as we have just listed them, indicate the unequal value of the applications, which we shall now review briefly.

PRINCIPAL APPLICATIONS OF IMMUNE-FLUORESCENCE

The applications of immune-fluorescence are very numerous and extremely varied. The technique was first used in basic research in immunology, virology, and bacteriology. Subsequently

it came into widespread use in the practical areas of medical diagnosis, particularly in infectious pathology.

I. -- APPLICATIONS IN BASIC RESEARCH

We shall cite only a few examples of this:

-- In immunology, the technique has enabled us to study the cellular sites of antibody synthesis. This was how the rôle of the plasmocytes was confirmed. Certain aspects of such phenomena as immunological paralysis were clarified.

Immune-fluorescence is also a practical tool for the study of so-called antibody affections, such as certain kinds of thyroid inflammations (Hashimoto's disease) or collagenoses. IF tests for antinuclear antibodies in systemic erythomatous lupus has even become a matter of routine. Still in the experimental stage, however, is the use of IF in testing for anti-adrenal cortex antibodies in cases of adrenal insufficiency, or antimucous antibodies in the stomach in cases of pernicious anemia, or of antimyocardic antibodies in rheumatic carditis. We should also mention possible uses of IF in colic, hepatic, renal, nervous, muscular, vascular, and sanguinary diseases, assumed to be autoimmune problems. The authors are still, and with good reason, leaning towards prudence in the interpretation of the results thus far obtained.

The rôle of local accumulations of immune-compounds as observed in certain diseases has been clarified; these include toxi-allergic, cutaneous and renal affections such as contact dermatitis and experimental toxic nephritis.

Tissue antigens can also be revealed by IF. Examples of this are hormones on the producing tissue level: pituitary hormones, insulin, glucagon, and thyroxin; and enzymes, such as trypsin. Specific antigens of normal organs can also be made visible, as well as the loss of this specificity in malign tumors. The live development of injected foreign antigens can also be studied, whether they be formed (bacteria) or non-formed (albumen, globulins, polysaccharides. The long persistence of these latter in the organism has been emphasized. The sensitization phenomena have also been observed and reported. One such example is the accumulation of antigens in the walls of blood vessels surrounding lesions was observed in the Arthus phenomenon.

-- In virology, applications of IF have also proved very fruitful. The visualization of viral antigens appearing in infected cells is a good means for studying the locale (cytoplasm or nucleus) and the chronology of viral reproduction.

Separate synthesis in the host cell of the various constituents of virus antigens can be observed.

Virus phagocytosis by leucocytes or macrophages has been demonstrated.

Several methods for counting virus particles, similar in principle to the measured field method, have been devised.

In experimental virus infections, it has made it possible to localize the pathogenic agent in the tissues.

The study of virus-induced tumors has also been furthered by IF.

-- In bacteriology, localization of antigens in the various structures of the bacterial cell has been studied. Other experimental work has been done on parasites, Rickettsia, fungi, etc.

II. -- PRACTICAL APPLICATIONS IN DIAGNOSIS OF INFECTIOUS DISEASES

These can be divided into two groups, according to whether immuno-fluorescence is used as a microbiological tool for finding the infectious agent in pathological products, or as a serological procedure in finding and titering the antibodies.

1º MICROBIOLOGICAL APPLICATIONS

1. -- Infantile gastro-enteritis caused by colibacilli.

Direct IF detection of pathogenic colibacilli in the stools is unquesti nably one of the best pract cal applications of the procedure.

Every author who has used it reports that it is more sensitive than the classical procedure of culture-agglutination, and just as specific. This high specificity is apparently due to the presence of the K-skin antigen (variety B) which surrounds the somatic antigen O.

The rapidity of the procedure makes it possible to single out the babies who are carriers of pathogenic colibacillus serum types upon their arrival in the hospital, or at the start of an epidemic.

2. -- Other intestinal infections

Attempts to use direct IF in research on Salmonella, Shigella, and Vibria comma, on the contrary, yielded poor results

because of the numerous cross-reactions with the saprophytic fecal enterobacteria.

3. -- Whooping-cough

Testing for Bordetella pertussis in smears of throat scrapings by direct IF is far faster than the method of culture on Bordet and Gengou media, whose results are, furthermore, uneven.

It can be of help at the beginning of the malady: it is, of course, at this point that it is most often positive.

However, errors of excess are possible by reason of the frequent difficulty of interpretation of smears from pharyngeal scrapings, where germs are often rare and non-specific fluorescents (cell elements, mucus, etc.) are frequent.

Even if you adopt very strict standards of interpretation, IF does not, in these cases, possess the reliability of the classical bacteriological examination.

4. -- Streptococcal infections

Direct IF is used in testing for group A streptococci in the pharynx in simple sore throat, scarlatina angina, RAA, etc. The time saved, as compared with the standard procedure, is considerable, since the latter involves isolation by freezing the blood and grouping by Lancefield serum precipitation. But here again, you run into problems of specificity. And anti-A conjugates must be subjected to attrition with C and G streptococci, in order to eliminate cross-reactions with those groups. And, on the other hand, you are never sure that you have really got rid of all cross-reactions with the staphylococci.

And so, in spite of repeated checks, in spite of the use of inhibition techniques, throat scrapings, because of the problems complicating those caused by non-specific fluorescence of non-bacterial elements in the scrapings, are difficult to interpret.

Use of preliminary incubation of the sample in a liquid medium, several hours before the scraping is taken, improves the results.

On the whole, however, this application of IF will need considerable improvement before it can be brought into general use with reliable results.

5. -- Diphtheria

Direct tests for Klebs-Loeffler bacilli in throat samples have been performed with two kinds of conjugates. One was a tagged antitoxin, and the other a solution of conjugated antimocrobial antibodies prepared from an animal that had been hyperimmunized with a freshly isolated strain which was antigenically complete.

The sensitivity of these conjugates leaves something to be desired, and their specificity is not really excellent in dealing with saprophyte Corynebacteria or other gram-positive germs.

This leaves us a long way from the accuracy of the classical (guinea-pig culture) procedure for bacteriological identification.

6. -- Purulent meningites

In the course of Pfeiffer bacillus meningites, the bacillus may be found in the LCR, even though it may no longer be viable following early treatment with antibiotics, and found with relative ease.

The Meningococci, the Pneumococci, and the Listeria can also be observed with IF. However, examination of an LCR in which the strain is in the pure state does not present the same problems encountered in examining a stool specimen, or particularly a throat specimen. This is why IF offers no practical advantage over the classic bacteriological culture procedures in this case.

7. -- Blennorrhea

IF has proven its worth particularly in subacute or chronic blennorrhea in women, where bacteriological examination is difficult and not particularly reliable. Results are perhaps slightly better than those yielded by the standard cultures of genital smears on special media, provided the sample is allowed to incubate for 16 to 20 hours.

In acute blennorrhea in men, IF is hardly worthwhile, since the usual procedures of staining are simpler and also yield satisfactory results.

8. -- Other bacterial infections

Suggested applications in infections with Staphylococcus, Pasteurella, Brucella, Leptospira, etc., have given no indication of any degree of superiority to the standard techniques.

9. -- Virus infections

In this area the practical applications are fairly limited at present. They involve both direct and indirect IF.

-- In rabies infections, IF provides identification of viral antigens in the cerebral or salivary glands of sacrificed animals, whether they die of the disease or are experimentally inoculated, with greater sensitivity than does the test for Negri bodies by histological staining.

-- Tests for flu viruses in the sloughed-off cells contained in gargle liquid are too often negative to be of any real interest.

-- Herpes viruses can be identified in samples taken from the eruptions.

-- Swift discovery of polio viruses by IF in cell cultures inoculated with pathological products makes it possible to avoid the lengthy findings of the cytopathogenic effect.

-- a more interesting technique recently suggested identifies the virus antigens in the leucocytes of the circulating blood during the acute phase of various viral infections. The smears are made with a concentrated solution of leucocytes. The intermediate layer consists of an experimental (rabbit) antiserum, and the second layer of an antigammaglobuline conjugate previously subjected to attrition by a suspension of human leucocytes to eliminate non-specific cellular fluorescence.

10. -- Parasitic and mycosic infections

Applications in this area are numerous, but they are still in the exploratory stages.

We believe it would be feasible to use IF in identifying the diverse varieties of amoebas, trypanosomes, schystosomes, trychomones,, etc.

Spontaneous fluorescence of mycelian elements, along with cross reactions, are problems that contribute to the delicacy of present-day applications to mycosic infections such as [illegible]didosis, histoplasmosis, etc.

2º SEROLOGICAL APPLICATIONS

In these cases the indirect method is always used, with a serum from the patient for the intermediate layer, and a conjugate of human antigammaglobulins for the second.

1. -- Syphilis

Use of IF in syphilis serology is fairly wid spread today. The antigen preparation consists of smears of Treponema pallidum taken from experimental rabbit orchitis (Nichols strain).

Meanwhile, along with the specific antibodies to the pathogenic Treponema, the reaction also reveals, as we have already pointed out, the group anti-protein common to pathogenic and non-pathogenic Treponema alike. The Reiter saprophyte strain may also be used as an antigen.

This detail would seem to explain a number of non-specific fluorescents observed in normal serums at weak dilutions. Using Reiter ultrasonats to purify patient serums seems better calculated to attenuate this disadvantage than does the choice of a significant dilution.

In any case, the only truly specific test today is still the Nelson test. This is where we must still turn when standard cardiac-lipid serology yields dubious or false results (although this is rare).

2. -- Atypical Eaton mycoplasm pneumonias

Indirect IF is invaluable in this infection, because all the bacteriological and other examinations are difficult to perform, or less sensitive. Spread of the method will unquestionably be hampered, however, by the difficulty of preparing the antigens. They consist of histological sections of the bronchopulmonary arch of embryo chicks, experimentally inoculated with Eaton's agent. If the patient's serum contains antibodies, the bronchial epithelium will be fluorescent. Repeated checks for specificity are necessary.

3. -- Toxoplasmosis

Indirect IF in this parasite infection reveals and titres serum antibodies with a sensitivity and specificity close to those provided by the Dye-test.

4. -- Other serological applications

These are numerous, but they are not yet in common use. Indirect IF can reveal serum antibodies in many infections: whooping-cough, leprosy, rickettsia, trypanosomiasis, etc.

CONCLUSION

The various Immune-fluorescent procedures are patient of numerous applications.

In the area of experimental research, they have enabled us to make some very valuable discoveries. In the area of practical applications, however, their potential is extremely varied, by reason of the numerous problems we have indicated. A number of these problems will undoubtedly be solved in the future. Meanwhile, it is well not to be too optimistic as to the utility of IF in microbiological or serological diagnosis of infectious diseases.

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